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2022.07.18 Project Skunkworks: Cloning Systems

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Creative Business Concept: the use of plasmid encoded chromogenic proteins for use as a selection marker for selecting a colony harboring a specific plasmid in molecular cloning procedures like TA cloning (1), Golden Gate and Golden-Gate-like DNA cloning (2), SLiCE molecular cloning (3), and traditional restriction enzyme/t4 ligase based DNA cloning (4). The plasmid backbone for all RGB vectors is a synthetic **pRadegenBio.pUC+** designed based on a modified pUC18 plasmid with a modular antibiotic selection marker.

1. Red-Green-Blue (RGB) Gate Cloning Plasmids

1.1. Cloning plasmids for DNA assembly using TypeIIS restriction enzymes. The system employs the use of 3 distinct cloning vectors ment for assembly using 3 distinct type IIS restriction enzymes. Each plasmid also codes for a specific chromogenic protein (5) designed for selection *E. coli* colony harboring a distinct vector. This system enables a reduction of consumables by providing the ability to combine distinct ligation reactions into one *E. coli* transformation reaction. Colonies obtained from the RGB transformation reactions are screened based on color. Red colonies harbor the BsaI plasmid, green colonies harbor the SapI plasmid and blue colonies harbor the BsmBI plasmid. Labs that spend tens of thousands of dollars USD on LB broth and agar plates can potentially reduce their cost by two thirds. RGB Gate Cloning Plasmids code for a Kanamycin resistance selection marker.

1.2. The system is specifically designed for simple cloning reactions with one plasmid, tiered based assembly of synthetic DNA from

duplexed oligonucleotides and synthetic DNA in various formats to complex braid assemblies.

1.2.1. pRadegenBio.pUC.rbRed.BsaI - a plasmid harboring a red chromogenic protein selection marker and a Multiple Cloning Site MCS containing the following general characteristics:

```
                                    BsaI->
5'-NNNNNNNNNNGAGACCNNNNNNNNNNGGTCTCNNNNNNNNN-3'
3'-NNNNNNNNNNCTCTGGNNNNNNNNNNCCAGAGNNNNNNNNN-5'
                 <-IasB
```

1.2.1.1. Red colored text is BsaI recognition site in opposing directions
1.2.1.2. Blue color text is MCU sequence flanked by opposing BsaI sites
1.2.1.3. Green colored text depicts the 4 bp 5' overhang retained by the vector backbone after BsaI digest

1.2.2. pRadegenBio.pUC.rbGreen.SapI - a plasmid harboring a green chromogenic protein selection marker and a MCS containing the following general characteristics:

```
                                    SapI->
5'-NNNNNNNNNgaagagcNNNNNNNNNNgctcttcNNNNNNNNN-3'
3'-NNNNNNNNNcttctcgNNNNNNNNNNcgagaagNNNNNNNNN-5'
                 <-IpaS
```

1.2.2.1. Green color text is SapI recognition site in opposing directions
1.2.2.2. Blue color text is MCU sequence flanked by opposing SapI sites
1.2.2.3. Red colored text depicts the 3 bp 5' overhang retained by the vector backbone after SapI digest

1.2.3. pRadegenBio.pUC.rbGFP.SapI - a plasmid harboring a green fluorescent protein selection marker. This plasmid is meant for use as a destination vector to harbor a cDNA sequence. The cDNA sequence should start with "TATG" followed by the second codon in the cDNA sequence. Digestion of this plasmid should be followed by gel purification. The cDNA fragment is then ligated into the 3 bp overhang sites on a linearized and gel purified OpSE MCS Plasmid produced by SapI digestion.

```
5' - GATTACAgctcttcaTATGcaccatcatcatcatcattctTCCCgaagagcGATTACA - 3'
3' - CTAATGTcgagaagtataCgtggtagtagtagtagtaagaAGGGcttctcgCTAATGT - 5'
```


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1.2.4. pRadegenBio.pUC.rbBlue.BsmBI - a plasmid harboring a blue chromogenic protein selection marker and a MCS containing the followng general characteristics:

```
                                BsmBI->
5'-NNNNNNNNNNgagacgNNNNNNNNNNcgtctcNNNNNNNNN-3'
3'-NNNNNNNNNNctctgcNNNNNNNNNNgcagagNNNNNNNNN-5'
                    <-IBmsB
```

1.2.4.1. Blue color text is BsmBI recognition site in opposing directions
1.2.4.2. Red color text is MCU sequence flanked by opposing BsmBI sites
1.2.4.3. Green color text depicts the 4bp 5' overhangs retained by the vector backbone after BsmBI digestion

2. *Ppu*λ red.cloning Plasmids

2.1. Cloning plasmid suite for use in SLiCE based cloning and DNA assembly. The suite consists of 3 plasmids with unique homology sites for tiered DNA assembly or for constructing a final expression plasmid for heterologous protein expression in *E. coli*. ***Ppu***λ red.cloning plasmids code for **Tetracycline** resistance selection marker. This plasmid suite comes with a N terminal tag option (2.1.1), a C terminal tag option (2.1.3), and a native expression option (2.1.2). These plasmid options do not have

2.1.1. RadegenBio+43 N - Terminal 6xHis

```
                                                        _T7 promoter______
5' -       tccggcgtag aggatcgaga tcgatctcga tcccgcgaaa ttaatacgac tcactatagg

_lac operator_________________                                _RBS____
ggaattgtga gcggataaca attcccctct agaaataatt ttgtttaact ttaagaagga gatatacat

_6x His Tag_____________________                                       ___
ATG CAC CAT CAT CAT CAT CAT TCT TCT GGT CTG GTG CCA CGC GGT TCT GGT ATG AAA
met his his his his his his ser ser gly leu val pro arg gly ser gly met lys

                                                                30 bp homology_
_______________S-Tag_ _____________________________________                ___
GAA ACC GCT GCT GCT AAA TTC GAA CGC CAG CAC ATG GAC AGC CCA GAT CTG GGT GAA
glu ser ala ala ala lys phe glu arg gln his met asp ser pro asp leu gly glu

_arm____________________                    __30 bp homology_arm__________
TEV_Clevage_________
AAC CTG TAC TTC CAG atg CGGTCTACGAAAGCACGGAT gacgacctgcagaatcgctggaaggccggc - 3'
Asn leu tyr phe gln|met
                  ^
```

2.1.2. RadegenBio+ Native Cloning

```
                __30 bp homology arm (-T7)____
                                                _T7 promoter______
5' -    tccggcgtag aggatcgaga tcgatctcga tcccgcgaaa ttaatacgac tcactatagg

                                                ___30 bp homology ___
        _lac operator_____________                              _RBS__
        ggaattgtga gcggataaca attcccctct agaaataatt Ttgtttaact ttaagaagga

        _arm_________                                            _

        gatatacaTA TG CGGTCTACGAAAGCACGGATGAATGCTGCTTCGGACCACG

        __30 bp homology_arm__________

        gacgacctgcagaatcgctggaaggccggc - 3'
```

2.1.3. RadegenBio+43 C - Terminal His Tag

```
                                                _T7 promoter______
5' -    tccggcgtag aggatcgaga tcgatctcga tcccgcgaaa ttaatacgac tcactatagg

                                                ___30 bp homology ___
        _lac operator_____________                              _RBS__
        ggaattgtga gcggataaca attcccctct agaaataatt Ttgtttaact ttaagaagga

        _arm_________
        _
        gatatacaTA TG CGGTCTACGAAAGCACGGATGAATGCTGCTTCGGACCACG

        ____30 bp homology arm_________________
        TEV_Clevage___________
        GAA AAC CTG TAC TTC CAG atg TCT TCT GGT CTG GTG CCA CGC GGT TCT


                ______________S-Tag_ ___________________________________
        GGT ATG AAA GAA ACC GCT GCT GCT AAA TTC GAA CGC CAG CAC ATG GAC


        ___                 6x His Tag_____________
        AGC CCA GAT CTG GGT CAC CAT CAT CAT CAT CAT TAA ggacgacctgc - 3'
```

3. OpenSource Enzyme MCS (OpSE) Plasmids (OpSE Plasmids)

3.1. Cloning plasmid suite containing the OpSE MCS. The multiple cloning site is designed to contain the restriction enzyme recognition site for Radegen Biotechnology's suite of restriction enzymes based on Open Source enzyme technology. The MCS is adjacent to an expression cassette meant for cloning a coding

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sequence by SapI digestion for IPTG inducible T7 expression. A donor plasmid or dsSynthDNA with the SapI digestion adapters can be used. **The cDNA sequence should start with "TATG" followed by the second codon in the cDNA sequence. A digestion adapter would be designed as follows and can be harbord on a synthetic dsDNA fragment or as (pRadegenBio.pUC.rbGFP.SapI):**

3.2. OpSE SapI T7 Expression Cloning Adapters

```
5' - GATTACAgctcttcaTATGcaccatcatcatcatcattctTCCCgaagagcGATTACA - 3'
3' - CTAATGTcgagaagtataCgtggtagtagtagtagtaagaAGGGcttctcgCTAATGT - 5'
```

3.3. OpSE - SapI T7 MCS - A multiple cloning site included in the **OpSE Plasmids suite**. This cloning plasmid is designed for versatility. There are several restriction enzyme sites that can be used for traditional recombineering that employs the use of primers to clone a fragment into a vector. This plasmid is specifically designed to construct a T7 IPTG inducible construct from a cDNA sequence either from a natural template or in a synthetic DNA format and designed with the **OpSE SapI T7 Expression Cloning Adapters.**

3.3.1. OpSE - SapI T7 MCS

```
                                                    _T7 promoter______
5' -    tccggcgtag aggatcgaga tcgatctcga tcccgcgaaa ttaatacgac tcactatagg

        _lac operator_____________                                  _RBS__
        ggaattgtga gcggataaca attcccctct agaaataatt Ttgtttaact ttaagaagga

        _           SapI     NotI    NcoI   HindIII  PstI     XbaI  EcoRI
        gatatacaTA TGGAAGAGCt gcggccgcCC ATGGaagctt atCTGCAGtt tctagaGAAT

          EcoRV    SpeI    SapI               SfiI
        TCgatatctt ACTAGTttGC TCTTCCCCCG ATTACAggcc tgtggccGAT TACA    - 3'
```

3.3.1.1. SfiI is considered a rare cutter with an 8bp recognition sequence and is ideal for plasmid linearization.

3.3.1.2. OpSe Plasmids code for an Ampicillin resistance selection marker.